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Research Article

Influence of exogenous salicylic acid on growth and biochemical parameters of *Spinacia oleracea L.* under salinity stress

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Abstract

Salinity stress is one of the most important abiotic stresses in arid and semi-arid climates that limit crop plants' growth and development. Salicylic acid (SA) is an endogenous plant growth regulator that can regulate physiological processes and improve the plant's tolerance to stress. A factorial experiment based on a completely randomized design was carried out to investigate the effects of different levels of SA (0, 0.5, and 1 mM) on some growth and biochemical parameters of spinach under salinity stress (0, 40, and 80 mM). Our findings showed that salinity negatively affected growth traits and photosynthetic pigments while SA increased them. For example, under severe salinity stress, a concentration of 1 mM SA increased shoot length by 23%, and 0.5 mM SA enhanced both the fresh and dry weight of the root by 26%. Also, under moderate and severe salinity stresses concentration of 1 mM SA increased shoot dry weight by 130 and 69%, shoot fresh weight by 52 and 42%, chlorophyll *a* by 53, and 86%, chlorophyll *b* by 79 and 112%, total chlorophyll by 63, and 96%, carotenoids by 63 and 64%, soluble sugars by 44 and 13%, anthocyanin by 48 and 25%, respectively in comparison to the control plants. In conclusion, a concentration of 1 mM SA decreased negative effects of salinity stress on evaluated growth and biochemical parameters more than 0.5 mM, and improved tolerance of the spinach plants to the salinity stress by an increase in plant growth, total chlorophyll and carotenoids, soluble sugars, and anthocyanin.

Keywords: Abiotic stress, Photosynthetic pigments, Salicylic acid, Spinach

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Introduction

Spinach (*Spinacia oleracea* L.) is a leaf vegetable belonging to the Amaranthaceae family that is rich in mineral elements and vitamins A, C, E, and phenolic compounds as flavonoids (Roberts and Moreau, 2016). The health-related effects of spinach include its antioxidant, anti-inflammatory, antitumoral, anti-obesity, and lipid-lowering effects in animal models and humans (Roberts and Moreau, 2016). Spinach is a salt-sensitive vegetable that tolerates salinity by 2 dS/m (Caparrotta *et al*., 2019). Plant growth, productivity, and yield are strongly affected by several biological and abiotic stresses all abiotic stresses, soil salinity is the most important limiting factor of agricultural productivity and food security (Shi-Ying *et al*., 2018; Singh *et al*., 2018). Generally, about 20% of agricultural lands are affected by salinity that increases continuously (Gupta and Huang, 2014). It is estimated that by 2050, about 50% of agricultural land will be affected by salinity due to the accumulation of salt in the soil (Bharti *et al*., 2016; Shi–Ying *et al*., 2018). High accumulation of Na^+ limits water conductivity, soil porosity, and aeration. Salinity leads to hypertensive stress due to the excessive accumulation of Na^+ and Cl^- ions. Also, high salt concentrations affect enzyme activity, stomata conductance, and photosynthesis (Kumar and Verma, 2018; Shi- Ying *et al*., 2018). In recent decades, develop strategies to improve the stress tolerance potential of plants (Yan *et al*., 2013). Salicylic acid is an essential phenolic compound widely distributed in the plant kingdom and regulates plant growth processes in response to biotic and abiotic stress (Wani *et al*., 2017; Fathi *et al*., 2019; Maruri-Lopez *et al*., 2019). Salicylic acid is a stress tolerance inducer and an important signal in many physiological processes, such as proline metabolism and photosynthesis that reduces oxidative stress in plants under environmental stress and increases plant growth and productivity under salinity stress (Rao, 2019). There is a positive correlation between salinity tolerance and

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endogenous levels of salicylic acid in the plant species (Gharbi *et al*., 2016; Liang *et al*., 2018). Exogenous application of salicylic acid has reduced the adverse effects of salinity stress on plant growth and improved biochemical parameters in the different plant species (Khan *et al*., 2014; Abdelaal, 2015; Hernandez-Ruiz and Arnao, 2018; Fathi *et al*., 2019). Iran is located in the arid and semi-arid region of the world, and about 15% of the total agricultural lands of Iran are affected by salinity. This experiment was conducted to optimally use saline land for spinach cultivation using salicylic acid as a moderator of the negative effects of salinity. The purpose of this research is to find the appropriate concentration of salicylic acid at different salinity levels as a factor in improvement plant growth under stress conditions.

Materials and methods

This research was done under greenhouse conditions (18-25 ºC, 12 h light, and 60-80% relative humidity) at Shahid Bakri Higher Education Center (Miandoab, Iran). To investigate the growth and physiological responses of spinach to salicylic acid (SA) under salinity stress, an experimental factorial was carried out in a completely randomized design with three replications. The effect of salicylic acid at three concentrations (0, 0.5, and 1 mM) and salinity at three levels (0, 40, and 80 mM NaCl or EC= 0, 3.6 and 7.2 dS/m and or control conditions, moderate and severe stress, respectively) on some growth and physiological parameters were investigated. The spinach seeds were collected from the local population of Miandoab city, Iran. Twenty seeds were planted in three-liter that filled with a mixture of clay, manure, and sand equally (1:1:1; v/v). In the first experiment, the soil EC (5.1 dS/m), pH (7.67), Na^+ (21.11 mg/l), Cl^- (22.67 mg/l), and HCO_3 (4.01 meq/L) were measured. Salinity was applied at the four-leaf stage of plant growth and salicylic acid leaf-sprayed twice (at the fourth leaf stage and three weeks later). At the end of the experiment, some morphological parameters, such as root length, shoot length, root dry weight, shoot dry weight, leaf number, leaf length, leaf width, and some physiological parameters, such as chlorophyll *a*, *b*, total chlorophyll, carotenoid, soluble sugars and anthocyanin were measured.

Measurement of some biochemical parameters: Chlorophyll and carotenoids were measured by Arnon method (1959), pigments were extracted using acetone 80% and solution absorption was measured by spectrophotometer at 645, 663, and 470 nm.

Soluble sugar was extracted by phenolic sulfur (Dubois *et al*., 1956) with 96% ethanol and measured by a spectrophotometer at 485 nm.

For anthocyanin determination, 50 mg of frozen tissue samples were crushed and homogenized in 5 mL of acidified methanol (methanol: HCl, 99:1 (v/v)) and was kept overnight at 25 ºC and in dark conditions. The extract was centrifuged at 4000 rpm for 10 min and absorption of the supernatant was read by a UV-VIS spectrophotometer at 550 nm. Anthocyanin content was calculated using an extinction coefficient of 33000 mol^{-1} cm^{-1} (Wagner, 1979).

Statistical analysis: Statistical analysis of the data was performed with SPSS software. Averages were compared by Duncan's method at the five percent probability level. The correlation between data was calculated using Minitab software.

Results

According to the variance analysis (Tables 1), the salinity and salicylic acid (SA) affected significantly ($P \leq 0.01$) all traits under study (Tables 2) interaction between the factors (salinity and SA) affected significantly all them (except leaf length and width the number of leaves) according to Duncan's test.

Growth parameters: Our result showed that severe (80 mM) salinity stress harmed all evaluated growth parameters (except the fresh and dry weight of root that increased in moderate (40 mM) salinity stress and total chlorophyll and carotenoids. The moderate (40 mM) and severe salinity stresses decreased root length by 33 and 20%, respectively compared to the control conditions (Fig. 1). The lowest root and shoot length belonged to untreated plants with salicylic acid r in the moderate and severe salinity stresses, respectively (Fig. 1). In both the control and salinity conditions, application of SA increased root and shoot length compared to sprayed plants with distilled water. The highest root and shoot length belonged to the sprayed plants with 0.5 and 1 mM SA under control conditions, respectively. In the severe salinity stress, application 1 mM salicylic acid had the best effect on shoot length and increased that by 23% compared to untreated plants with salicylic acid (Fig. 1).

Our findings showed, the fresh and dry weight of root under moderate salinity stress increased by 22 and 32%, respectively, and under severe salinity stress decreased by 21% compared to the control plants (Fig. 2). Application 0.5 mM SA increased both fresh and dry weight of root by 26% under severe salinity stress compared to sprayed plants with distilled water.

Proportionally with an increase in the salinity levels, the fresh and dry weight of the shoot decreased significantly compared to the control plants. Under moderate and severe salinity stresses, the shoot dry weight decreased by 34 and 70%, and the shoot fresh weight decreased by 10 and 52%, respectively compared to the control plants. The concentration of 1 mM SA under moderate and severe salinity stresses increased shoot dry weight by 130 and 69%, and shoot fresh weight by 52 and 42%, respectively compared to the sprayed plants with distilled water (Fig. 3).

An increase in salinity concentration reduced leaf length, leaf width, and increased leaf number. There was no a significant difference between concentrations of 40 and 80-mM salinity in both leaf width and leaf number (Table 2). Salicylic acid concentrations (0.5 and

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Table 1. Variance analysis of the effect of salinity and salicylic acid on some growth parameters

Sources of variations	df	Root Length	Shoot Length	Root Dry Weight	Shoot Dry Weight	Leaf Length	Leaf Width	Leaf Number
Salinity	2	102.27**	73.53**	11.69**	5.46**	1.74**	1.19**	75.11**
Salicylic acid (SA)	2	29.10**	27.89**	1.26**	1.18**	0.47**	0.89**	24.11**
Repeat	2	1.44ns	0.74ns	0.11ns	0.01ns	0.03ns	0.01ns	0.77ns
Salinity×SA	4	4.86**	8.67**	0.32*	1.33**	0.12ns	0.06ns	3.89 ns
Error	18	0.87	1.49	0.08	0.02	0.06	0.03	3.15
C.V.	-	4.33	7.11	5.75	9.45	3.41	4.95	5.72

ns, * and **, non- significant and significant at 5% and 1% level of probability

Table 2. Simple effect of salinity stress and salicylic acid on leaf length, leaf width and leaf number in spinach

Salinity stress (mM)	Leaf length (cm)	Leaf width (cm)	Leaf number
0	7.56 a	3.81 b	29.11 b
40	7.21 b	3.81 a	34.00 a
80	6.68 c	3.15 a	34.22 a
Salicylic acid (mM)			
0	6.9 b	3.24 b	30.66 b
0.5	7.19 a	3.69 a	32.77 a
1	7.36 a	3.81 a	33.88 a

The different letters at the top of the columns indicate a significant difference (P≤0.05) according to Duncan's multiple range test.

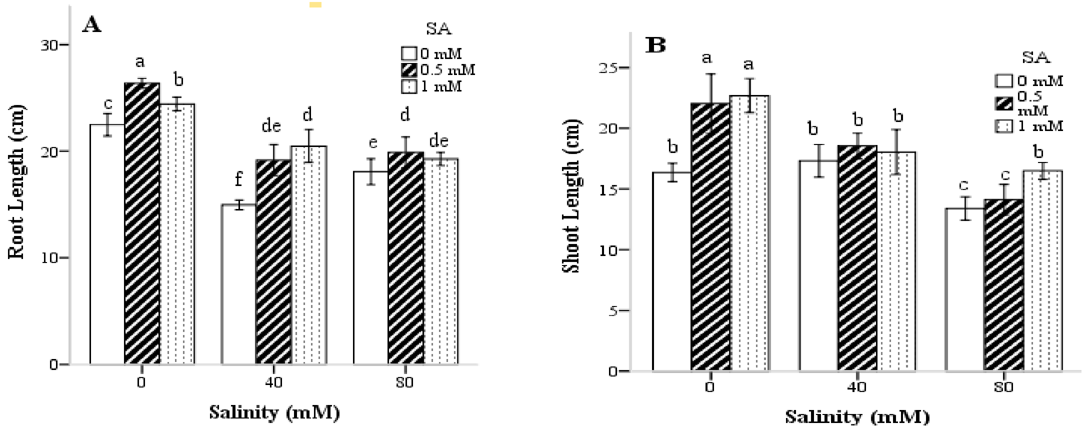

Fig. 1. Effect of salicylic acid foliar application on root length (A) and shoot length (B) in spinach under salinity stress. The different letters at the top of the columns indicate a significant difference (P≤0.05) according to Duncan's multiple range test.

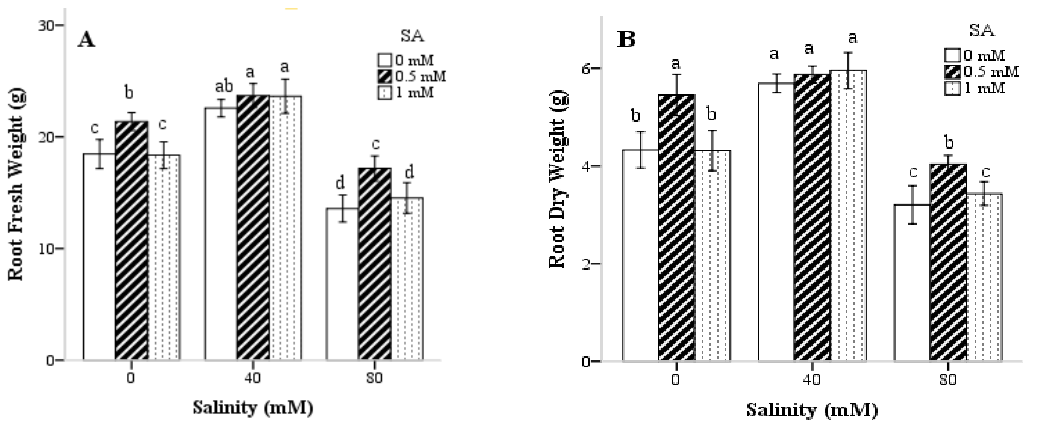

Fig. 2. Effect of salicylic acid foliar application on root fresh weight (A) and root dry weight (B) in spinach under salinity stress. The different letters at the top of the columns indicate a significant difference (P≤0.05) according to Duncan's multiple range test.

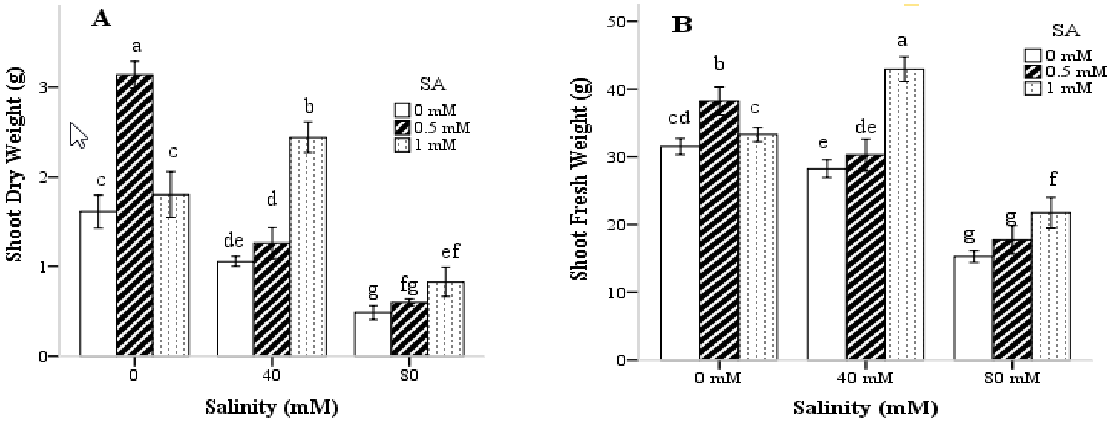

Fig. 3. Effect of salicylic acid foliar application on shoot dry weight (A) and shoot fresh weight (B) in spinach under salinity stress. The different letters at the top of the columns indicate a significant difference (P≤0.05) according to Duncan's multiple range test.

Table 3. Variance analysis of the effect of salinity and salicylic acid on some biochemical parameters

Sources of variation	df	Chl *a*	Chl *b*	Total Chlorophyll	Carotenoid	Soluble Sugars	Anthocyanin
Salinity	2	0.65**	0.35**	0.11**	13.39**	103.27**	0.00**
Salicylic acid (SA)	2	0.57**	0.59**	2.28**	47.83**	140.54**	0.00**
Repeat	2	0.001ns	0.002ns	0.006ns	0.005ns	1.635ns	0.000ns
Salinity × SA	4	0.04**	0.03**	0.05**	1.93**	23.52**	4.68**
Error	18	0.00	0.00	0.00	0.42	1.61	0.013
C.V	-	4.64	5.07	5.06	6.21	6.24	5.84

ns, and **, non- significant and significant at 1% level of probability

1 mM) increased significantly leaf length, leaf width, and leaf number (Table 3). There was a positive and significant correlation between leaf numbers and root fresh weight, root dry weight, shoot fresh weight, shoot dry weight, leaf length, and leaf width (Table 4).

Photosynthetic pigments: Salinity, salicylic acid levels, and interaction between them affected significantly chlorophyll a, b, total, and carotenoid (Table 3). The increase in salinity levels decreased the content of total chlorophyll and carotenoid compared to the control (Fig. 4). Under the control and saline conditions, the salicylic acid concentrations increased significantly the content of photosynthetic pigments compared to sprayed plants with distilled water. Proportionally the increase in the concentration of salicylic acid increased photosynthetic pigments. Application of 1 mM salicylic acid under control, moderate and severe stresses increased chlorophyll *a* by 86, 53, and 86%, chlorophyll *b* by 131, 79, and 112%, and total chlorophyll by 104, 63, and 96%, carotenoids by 47, 63 and 64%, respectively in compared to the sprayed plants with distilled water (Fig. 4). There was a significant positive correlation (R=0.783**) between chlorophyll *a* and *b* and (R=0.918**) between chlorophyll *a* and carotenoid (Table 3).

Soluble sugars and anthocyanin: Salinity increased significantly the content of soluble sugars and anthocyanin. Moderate and severe salinity stresses increased soluble sugars by 56 and 118%, and anthocyanin by 66 and 151%, respectively compared to the control condition. Application of 1 mM salicylic acid under moderate and severe salinity stresses, increased soluble sugars by 44 and 13% compared to sprayed plants with distilled water. Application of 0.5 and 1 mM salicylic acid under moderate salinity conditions increased anthocyanin by 40 and 48% and application of 1 mM salicylic acid under severe salinity conditions increased anthocyanin by 25% compared to sprayed plants with distilled water (Fig. 5).

Concentration of 1 mM salicylic acid increasedsoluble sugars and anthocyanin contents in compared to 0.5 mM concentration. According to table 4, there was a positive and significant correlation between soluble sugars and total chlorophyll (R=0.897**) and between soluble sugars and carotenoids (R=900**).

Discussion

According to our results, some morphological and growth parameters such as shoot length, leaf number, and fresh and dry weight of root increased in moderate salinity stress compared to the control conditions. While the severe (80 mM) salinity stress led to a significant reduction in fresh and dry weight of the shoot, leaf length, leaf width, and total chlorophyll and carotenoid contents. It seems an increase in the some growth parameters under the moderate (40 mM) salinity stress is caused by the natural adaptation of this plant to fairly saline environments, which increases its ability to remediate salinity. These results agree with the findings of Sogony *et al*. (2021) were reported total yield and

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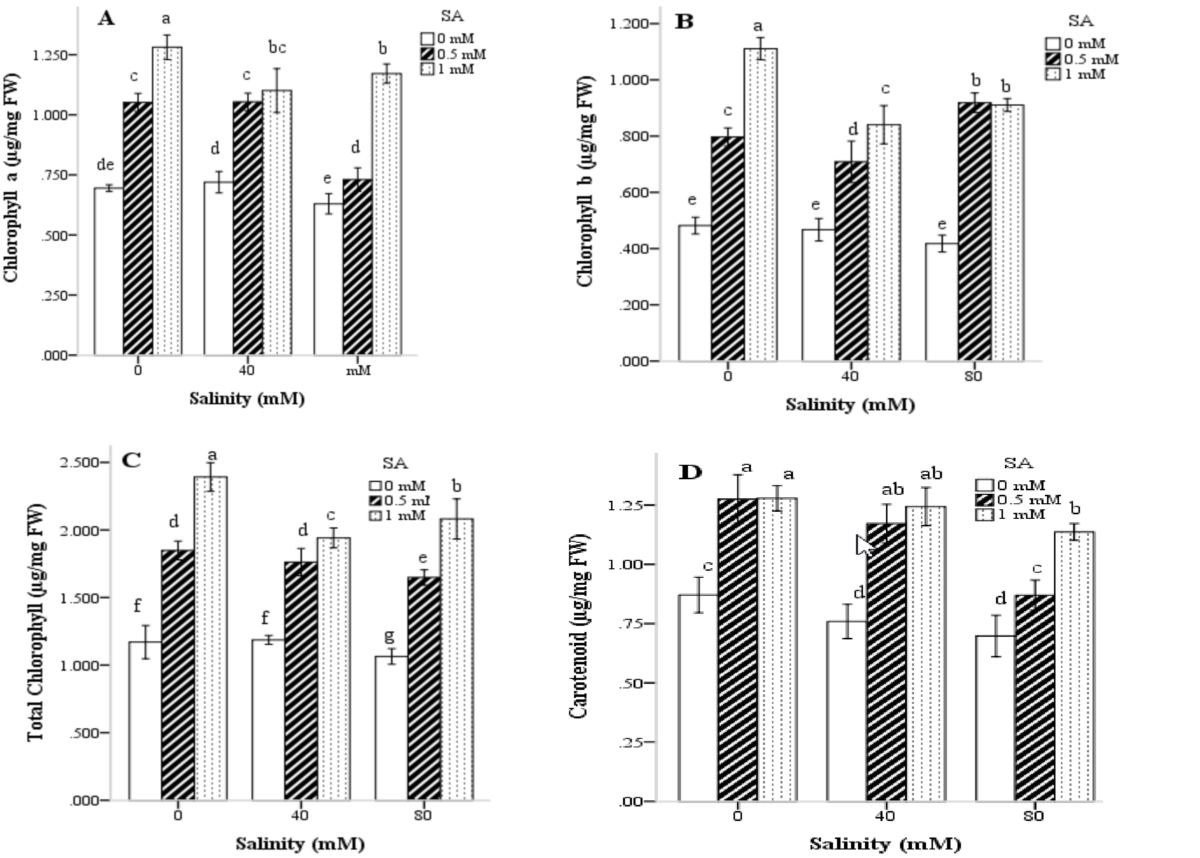

Fig. 4. Effect of salicylic acid foliar application on chlorophyll *a* (A), chlorophyll *b* (B), total chlorophyll (C) and carotenoids (D) in spinach under salinity stress. The different letters at the top of the columns indicate a significant difference (P≤0.05) according to Duncan's multiple range test.

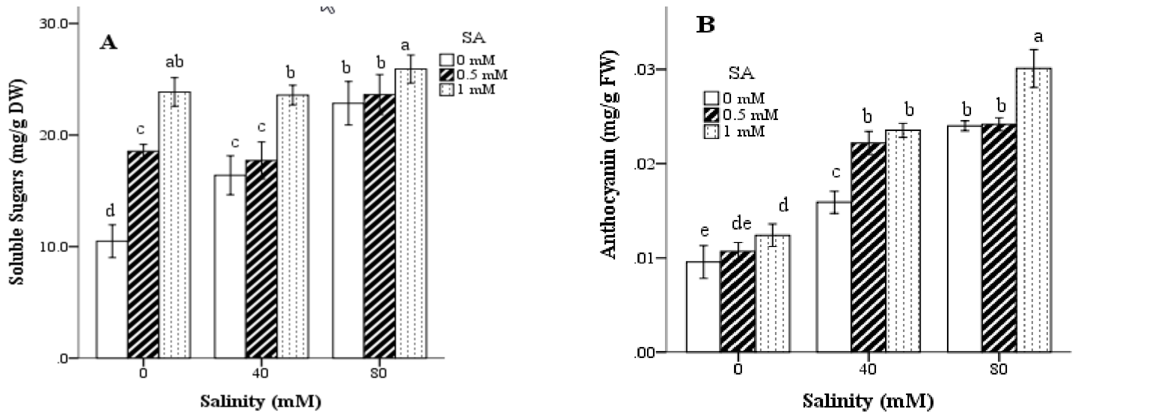

Fig. 5. Effect of salicylic acid foliar application on total soluble sugar (A) and anthocyanin (B) in spinach under salinity stress. The different letters at the top of the columns indicate a significant difference (P≤0.05) according to Duncan's multiple range test.

brunch production of dune spinach irrigated with 50 mM NaCl in the nutrient solution, increased significantly compared to the control. The ability of spinach to tolerate these varying salinity concentrations could be attributed to osmotic, ion, and tissue tolerance. It has been reported in numerous, an increase in salinity negatively affected plant growth parameters (Guo *et al*., 2018; Rahneshan *et al*., 2018; Sogony *et al*., 2021). Therefore, it seems that this plant tolerates salinity in 40 mM NaCl partially. A decrease in leaf length, leaf width, and leaf number in this study under salinity conditions (80 mM) may be due to an increase in abscisic acid under stress conditions. Abscisic acid is an important phytohormone that increases root and leaf under salinity stress to increase plant incompatibility to stress conditions and it antagonistically regulates salicylic acid-mediated defense signaling (Robert-Seilaniantz *et al*., 2011; Gupta and Huang, 2014). Salicylic acid is an important mediator molecule in plants' response to environmental stresses, which increases cell division within meristem tissue and improves plant growth (Shakirova *et al*., 2003;

Table 5- Pearson correlation coefficients of spinach measurement traits under salinity stress condition and salicylic acid foliar application.

Parameter	1	2	3	4	5	6	7	8	9	10	11	12	13
1. Root length	1												
2. Shoot length	0.620**	1											
3. Root dry weight	0.010	0.444*	1										
4. Shoot dry weight	0.710**	0.734**	0.584**	1									
5. Leaf length	0.590**	0.744**	0.552**	0.728**	1								
6. Leaf width	0.550**	0.833**	0.672**	0.762**	0.816**	1							
7. Leaf number	0.430*	0.702**	0.709**	0.763**	0.696**	0.783**	1						
8. Chlll *a*	0.470 *	0.707**	0.212	0.469*	0.461*	0.632**	0.456*						
9. Chl *b*	0.470*	0.472*	-0.020	0.260	0.331	0.506**	0.281	0.780**	1				
10. Chl *t*	0.490**	0.611**	0.102	0.385*	0.394*	0.589**	0.377	0.930**	0.940**	1			
11. Car	0.630**	0.735**	0.356	0.676**	0.617**	0.757**	0.614**	0.920**	0.730**	0.862**	1		
12. Soluble sugars	0.770*	-0.427	0.634	-0.214	0.244	0.148	0.150	0.950**	0.810**	0.897**	0.900**	1	
13. Anthocyanin	-0.532*	-0.53**	-0.320	-0.297	-0.603	-0.408*	-0.464*	0.062	0.134	0.103	-0.91	0.667**	1

*** and ** show significant at 5% and 1% level of probability respectively. Chl: Chlorophyll, Chl *t* : Chlorophyll total and Caro: Carotenoid**

Mandhanis *et al.*, 2006). The use of salicylic acid on spinach improved all studied traits in both control and salt stress conditions. Also, an increase in plant growth by salicylic acid treatment can be due to an increase in biosynthesis and transport of IAA that is regulated by salicylic acid (Pasternak *et al.*, 2019) because, both salicylic acid and IAA are also produced by the shikimate biosynthetic pathway (Perez-Llorca *et al.*, 2019). The endogenous level of salicylic acid increases along with the activity of salicylic acid biosynthetic enzyme under salinity stress (Sawada *et al*., 2006). It has been reported that salicylic acid improved salinity tolerance by reducing the accumulation of Na^+ in the shoots not preventing of accumulation of Na^+ in roots (Jayakannan *et al.*, 2013). Photosynthetic pigments (chlorophyll *a*, *b*, total, and carotenoids) significantly decreased in salinity-stressed plants. The greatest content of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids were obtained in sprayed plants with 1 mM salicylic acid under salinity stress. Decreasing chlorophyll synthesis has been reported in different species of plants under salinity stress (Khan *et al*., 2014; Sogoni, 2021). The chloroplast membrane is composed of two phospholipid layers. Superoxide radicals produced during salinity stress cause lipid peroxidation (Hameed *et al*., 2021). It has been reported that the photosynthetic pigments under salinity stress could be due to the destruction of chloroplast, increase of chlorophyllase enzyme, inhibition of new chlorophyll biosynthesis, and reduction of minerals uptake directly influences photosynthetic functioning (Shams *et al*., 2019). Salicylic acid protects the plant against oxidative stress by reducing lipid peroxidation through enzymatic and non-enzymatic defense mechanisms. Our results are in agreement with the findings of previous research that has reported salicylic acid to prevent salinity-induced photosynthetic arrest in *Vicia faba* (Souana *et al*., 2020).

In this research, salinity decreased slightly in carotenoid content and salicylic acid application has a positive effect on carotenoids in control and stress conditions. It is well-distinguished that carotenoids can protect the photosynthetic apparatus from damage by reactive oxygen molecules. Carotenoids protect the plant against oxidative stress by sweeping free radicals (Padash *et al*., 2019). From a recent study, it can be concluded that improved photosynthesis due to salicylic acid and no application may have resulted due to the up-regulation of antioxidant and osmolyte accumulation leading to lesser reactive oxygen species accumulation and maintenance of tissue water content, respectively (Ahanger *et al*., 2017). The content of soluble sugars and anthocyanin increased significantly with increasing salinity and the application of the salicylic acid concentrations improved significantly the content of soluble sugars and anthocyanin. The main role of these carbohydrates in reducing stress includes protecting the osmosis, storing carbon, and eliminating reactive oxygen species. It was observed that salt stress increases sugar (sucrose and fructan) inside the cell in the number of plants belonging to different species (Khoshbakht *et al*., 2012; Guptan and Huang, 2014). Also, Khoshbakht *et al*. in 2012 stated the content of soluble sugars increased under salinity stress in beans and the use of salicylic acid caused the metabolic consumption of sugars soluble in new cell compounds, as a mechanism to increase growth under salinity stress. The adaptability of plant species to high salinity concentrations in the soil is associated with a decrease in the osmotic potential of the tissue due to the accumulation of solutes (Azooz *et al*., 2009). Increasing anthocyanin content in high levels of salinity is a kind of defense response to this abiotic environmental stress. In addition, anthocyanin acts as an antioxidant and an osmotic regulator. Anthocyanin is a flavonoid whose

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biosynthetic pathway is controlled by environmental conditions, and under stress conditions occur synthesis of anthocyanin (Horbowicz *et al*., 2008). Therefore, our findings confirm the potential application of salicylic acid to improve the growth and benefits of *Spinacia oleracea* against salinity stress.

Conclusion

With an increase in the salinity levels, fresh and dry weight of shoot, leaf length, leaf width, total chlorophyll, and carotenoids contents decreased gradually, and soluble sugars and anthocyanin contents increased significantly compared to the control plants. Spinach plants responded to a partial adaptation to moderate salinity stress by an increase in the root fresh and dry weight compared to the control conditions. Application of salicylic acid (0.5 and 1 mM) could enhance spinach tolerance to the severe salinity stress due to an increase in growth parameters, photosynthesis pigments, total soluble sugar, and anthocyanin. Therefore, the application of salicylic acid (especially 1-mM) could further improve spinach cultivation in the moderate and severe salinity conditions. This research could provide useful information for the application of salicylic acid when cultivating spinach in moderate (40 mM NaCl) and severe (80 mM NaCl) salinity stresses.

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